THERAPEUTIC COMPOSITIONS (II).

The present invention relates to compositions suitable for administration to humans and animals which have the properties of increasing levels of (R)-3-hydroxybutyrate ((R)-3-hydroxybutyric acid or D-β-hydroxybutyrate) when so administered; particularly when administered orally, topically, subcutaneously or parenterally, but most advantageously orally.

Administration of (R)-3-hydroxybutyric acid has a number of beneficial actions on the human and animal body. These include *inter alia*, increasing cardiac efficiency, eg. in heart failure, provision of an alternative energy source to glucose, eg. in diabetes and insulin resistant states, and treating disorders caused by damage to neuronal cells, eg. CNS cells, particularly by retarding or preventing brain damage such as found in Alzheimer's and Parkinsonism and similar diseases and conditions.

Sodium hydroxybutyrate has been shown to increase cerebral circulation and regional vasomotor reflexes by up to 40% (Biull.Eksp.Biol.Med Vol 88 11, pp555-557). EP 0780123 A1 further teaches use of acetoacetate, β-hydroxybutyrate, monohydric, dihydric or trihydric alcohol esters of these or linear oligomers of 2 to 10 repeats of β-hydroxybutyrate for suppressing cerebral edema, protecting cerebral function, rectifying cerebral energy metabolism and reducing the extent of cerebral infarction.

Intravenous infusion of sodium salts of (R)-3-hydroxybutyrate has been performed on normal human subjects and patients for a number of conditions, eg. those undergoing treatment for severe sepsis in an intensive care unit and is found to be non-toxic and capable of decreasing glucose free fatty acids and glycerol concentration, but ineffective in decreasing leucine oxidation.

The present inventor has further determined that compounds and compositions that raise blood levels of (R)-3-hydroxybutyric acid and/or acetoacetate also have utility in reducing free radicals *in vivo*, and thus have a place in treatment of free radical associated diseases.

(R)-3-hydroxybutyrate and acetoacetate, commonly referred to as ketone bodies, provide a normal physiological alternative to the usual energy producing substrates, glucose and fatty acids. During prolonged fasting in man fatty acids are converted by liver to (R)-3-hydroxybutyric acid and acetoacetate which can be utilized by most major tissues of the body except liver.. Under these conditions, total blood ketone bodies are

elevated to about 7 mM. When these are modestly elevated in the blood, extrahepatic tissues such as brain, heart and skeletal muscle utilize these ketone bodies within the mitochondria to provide reducing power in the form of NADH which is the primary substrate of the electron transport system and generator of the energy required for the synthesis of ATP. In turn, generation of mitochondrial NADH by ketones, lowers the ratio of free mitochondrial [NAD^+] /[NADH] ratio and the cytosolic [$NADP^+$]/[NADPH] ratio to which the mitochondrial [NAD^+]/[NADH] is linked. While the catabolism of ketones reduces mitochondrial [NAD^+]/[NADH] it oxidizes the ratio of mitochondrial [ubiquinone]/[ubiquinol], [Q]/[QH_2]. The semiquinone form of ubiquinol is the major source of the generation by mitochondria of superoxide, O_2^-. By decreasing the amount of the reduced form QH_2, and its semiquinone, one can decrease the generation of free radicals by mitochondria while at the same time increasing the scavangers of free radicals linked to the NADP system, such as glutathione.

The inventor has thus determined that free radical damage resulting from excess reduced Q or inhibition of NADH dehydrogenase, such as occurs in MPP induced toxicity, can be reduced by administration of agents which elevate ketone body levels *in vivo*.

A number of disease processes involve damage by free radicals among which are the neurological diseases: Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease and cerebral ischemia. In addition excessive free radical damage has been implicated as playing a role in coronary reperfusion, diabetic angiopathy, inflammatory bowel disease and pancreatitis.

The inventor's copending WO 98/41201 discloses the administration of linear esters of (R)-3-hydroxybutyric acid and/or acetoacetate in producing elevated levels of the free compounds in vivo. Oral administration of 4mM solutions of the oligomer tetra-(R)-3-hydroxybutyrate or its acetoacetyl ester was shown to raise blood levels of ketone bodies such that (R)-3-hydroxybutyrate levels could be measured to have increased by 1 to 2 mM for periods in excess of 2 hours.

The inventor has now determined that unexpected advantages are provided when the (R)-3-hydroxybutyric acid component of such composition is administered as a cyclic oligomer. These advantages may include, *inter alia*, (a) increased efficiency in raising blood (R)-3-hydroxybutyric acid levels such that levels may be increased by

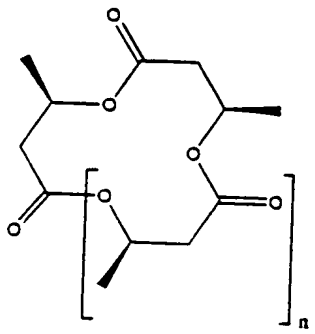
more than 2 mM, including attainment of near fasting levels and beyond, (b) maintenance of elevated levels for periods of several hours, (c) ability to be administered without counterion, such as sodium or methylglucamine, where it is desirable not to increase a patient's salt load or where significant dosing is envisaged and (d) relative ease of manufacture of pure compound from polymeric starting materials available through bioculture.

The present application particularly addresses the problem of neurodegenerative diseases, particularly disease where neurons are subject to neurotoxic effects of pathogenic agents such as protein plaques and oxidative damage and further provides compositions for use in treating these and the aforesaid disorders.

In preferred embodiments the present invention provides elevation of blood ketones necessary to correct the defects described above and can be accomplished by parenteral or enteral administration. Particularly it does not require the administration of potentially toxic pharmacological agents. The present invention's improved efficacy in raising levels, particularly blood levels, of ketone bodies provides therapeutic effects of the classical ketogenic diet, which is not itself found to be toxic in children, with none of the side effects that render that unused adults. Furthermore, the inventor has determined that with the correction of the aforesaid metabolic and toxic defects, cytokine responses and the increase in apoptotic peptides in degenerating cells will decrease due to the increase in neuronal cell energy status and the increased trophic stimulation resulting from increased neurotransmitter, eg. acetyl choline, synthesis.

The treatment that the present inventor provides goes beyond ketone body effects on circulation, as it provides treatment for cells that are unable to function due to neuro-degeneration and/or metabolic defects, particularly in metabolism of glucose, eg caused by neurotoxic agents such as peptides, proteins, free radical damage and effect of genetic abnormality. The treatment involves action of ketone bodies on the cells themselves and not the flow of blood to them.

Thus in a first aspect of the present invention there is provided a cyclic ester of (R)-3-hydroxybutyrate of formula (I)



where n is an integer of 1 or more

or a complex thereof with one or more cations or a salt thereof for use in therapy or nutrition.

For oral delivery free cyclic oligomer may be preferred. Where cations are present in a complex preferred cations are sodium, potassium, magnesium and calcium. and are balanced by physiologically acceptable counter-anion providing a salt complex.

Examples of typical physiologically acceptable salts will be selected from sodium, potassium, magnesium, L-Lysine and L-arginine or eg. more complex salts such as those of methyl glucamine salts

Preferably n is an integer from 1 to 200, more preferably from 1 to 20, most preferably from 1 to 10 and particularly conveniently is 1, ie. (R, R, R)-4, 8, 12-trimethyl-1, 5, 9-trioxadodeca-2, 6, 10-trione, 2, 3, 4 or 5.

The cyclic esters of the invention are preferably used in the treatment of disease states mediated by free radicals, toxic agents such as peptides and proteins, genetic defects detrimental to nerve cell metabolism, insulin resistance or other glucose metabolism defects or defect inducing states, ishemia, head trauma and/or for increasing cell efficiency, eg. cardiac cell efficiency eg. in heart failure.

A second aspect of the invention provides methods of treating cells that are subject to malfunction due to action of free radicals, toxic agents such as peptides and proteins, genetic defects detrimental to cell metabolism, insulin resistance or other glucose metabolism defects or defect inducing states, ischemia, head trauma and/or for increasing cell efficiency characterised in that it comprises administration of a cyclic oligomer of formula (I). This may include treatment of such disease states in humans and/or animals.

This aspect includes such use as a neuronal stimulant eg capable of stimulating axonal and/or dendritic growth in nerve cells, eg. in hippocampal or substantia nigral cells, *in vivo* or *in vitro*, particularly in conditions where neurodegeneration has serious clinical consequences, through its elevating effect on blood and plasma (R)-3-hydroxybutyrate and acetoacetate levels.

A third aspect of the invention provides a method of enteral or parenteral nutrition, preferably oral route nutrition, comprising administration of a cyclic oligomer of formula (I).

A fourth aspect of the invention provides the use of a cyclic ester formula I for the manufacture of a medicament for the treatment of disease states mediated by free radicals, toxic agents such as peptides and proteins, genetic defects detrimental to cell metabolism, insulin resistance or other glucose metabolism defects or defect inducing states, ischemia, head trauma and/or for increasing cell efficiency.

A fifth aspect of the invention provides composition characterised in that it comprises a cyclic oligomer of formula (I) in physiologically acceptable form eg. with a physiologically acceptable carrier.

Particularly the composition is suitable for parenteral or enteral administration, particularly for oral administration. Where the composition is for parenteral use it is sterile and pyrogen free. For oral use the composition may include a foodstuff base and may be in the form of an emulsion or mere admixture with solid food.

Particularly the cyclic oligomer(s) comprise an effective amount of the total composition, eg. at least 2% or more, eg at least 5%, of the composition by weight, more preferably 20% or more and most preferably 50% to 100%. The composition may be adapted for oral, parenteral or any other conventional form of administration.

In preferred forms of all of the aspects of the invention the compound of formula (I) is administered together with a physiological ratio of acetoacetate or a metabolic precursor of acetoacetate. The term metabolic precursor thereof particularly relates to compounds that incorporate acetoacetyl moieties such as acetoacetyl-1,3-butandiol, preferably acetoacetyl-(R)-1,3-butandiol, acetoacetyl-(R)-3-hydroxybutyrate, and acetoacetylglycerol. Esters of any such compounds with monohydric, dihydric or trihydric or higher, eg. glucosyl, alcohols are also envisaged.

In diabetic patients this use of the cyclic oligomers allows maintenance of low blood sugar levels without fear of hypoglycemic complications. In normal non-diabetic subjects the fasting blood sugar is 80 to 90 mg % (4.4-5mM) rising to 130mg % (7.2mM) after a meal. In diabetics 'tight control' of diabetes has long been recommended as a method for retardation of vascular complications but, in practice, physicians have found it difficult to keep blood sugars tightly controlled below 150mg % (8.3 mM) after eating because of hypoglycaemic episodes. Hypoglycaemic coma occurs regularly in normal subjects whose blood sugar drops to 2mM. As discussed earlier, (62, 63) in the presence of 5mM blood ketones there are no neurological symptoms when blood sugars fall to below 1mM.

The present inventor has determined that supplementing type II diabetics with cyclic oligomers of the invention will allow better control of blood sugar, thus preventing the vascular changes in eye and kidney which occur now after 20 years of diabetes and which are the major cause of morbidity and mortality in diabetics.

Where the therapy is aimed at seizure related disorders, such as refractory epilepsy as is treated by the ketogenic diet, therapy is improved by use of cyclic oligomers, due to the reduction or elimination of both high lipid and carbohydrate content. Such patients include those with genetic defects in the brain glucose transporter system, in glycolysis or in PDH itself such as in Leigh's syndrome, endotoxic shock or high stress states.

Particular disorders treatable with these medicaments are applicable to all conditions involving PDH blockage, including those conditions occuring after head trauma, or involving reduction or elimination of acetyl CoA supply to the mitochondrion such as insulin coma and hypoglycaemia, defects in the glucose transporter in the brain, or elsewhere (80), or in glycolytic enzyme steps.

Where the medicament or nutraceutical comprises acetoacetate it is preferably not stored for a prolonged period or exposed to temperatures in excess of 40°C. Acetoacetate is unstable on heating and decomposes violently at 100°C into acetone and CO_2. In such circumstances it is preferred that acetoacetate is generated by the composition on contact with the bodies metabolic processes. Preferably the composition comprises an ester precursor of actetoacetate.

A sixth aspect of the invention provides a method of treating a human or animal neuronal cell, eg. brain cells, subject to cell damage related disorder, particularly those which lead to cell death, as referred to for the second to fourth aspects, particularly a neurodegenerative disorder eg. such as those related to neurotoxic conditions such as presence of amyloid protein, eg. a memory or movement associated disorder such as Alzheimer's or Parkinson's diseases, or epileptic seizures, comprising administering to that person at least one of the materials for use in the first to fifth aspects of the invention.

The inventor has further determined that ketone bodies, provided by administration of the cyclic oligomers of (R)-3-hydroxybutyric acid in amounts sufficient to raise total blood ketone body concentration to elevated levels result in more than simple maintenance of cell viability but actually improve cell function and growth beyond that of normal, ie. control levels in a manner unrelated to blood flow or nutrition. In this respect the invention further provides use of the cyclic oligomers as agents capable of producing neuronal stimulation, ie. nerve growth factor like activity, increase of metabolic rate and increase of extent of functional features such as axons and dendrites. This aspect of the present invention offers a mechanism for improvement of neuronal function as well as mere retardation of degredation.

The recent work of Hoshi and collaborators (77, 78) strongly suggests that a part of the amyloid protein whose accumulation is the hallmark of Alzheimer's disease, $A\beta_{1-42}$, acts to stimulate mitochondrial histidine protein kinase which phosphorylates and inactivates the pyruvate dehydrogenase multienzyme complex. The PDH complex is a mitochondrial enzyme responsible for the generation of acetyl CoA and NADH from the pyruvate produced by glycolysis within the cytoplasm. The mitochondrial acetyl CoA formed condenses with oxaloacetate to start the Krebs TCA cycle completely combusting pyruvate to CO_2 while providing the mitochondria with the reducing power which becomes the substrate for the electron transport system through which the energy required for mitochondrial ATP synthesis is generated

Ketone body utilization in brain is limited by the transport, with lesser utilization occurring in the basal ganglion at blood levels below 1 mM (76). However, at levels of 7.5 mM achieved in normal man by prolonged fasting, the rate of ketone body entry into brain is sufficient to take over the majority of cerebral energy needs and to prevent

hypoglycemic symptoms, even in the face of blood sugar levels which would normally cause convulsions or coma (63) .

In the copending application WO 98/41201, 'Therapeutic compositions', it is the inventor's hypothesis that in Alzheimer's disease, where there is a block at PDH which prevents the normal energy production from glucose, if one can provide elevated, eg. normal fasting levels of ketones, one can bypass the PDH blockade present in these patients thereby preventing cell death due to energy depletion or lack of cholinergic stimulation and thus slow the progression of the memory loss and dementia. Furthermore, utilising the nerve growth/stimulatory effects of the ketone bodies, particularly (R)-3-hydroxybutyrate or a physiological ratio of this with acetoacetate, cells that are still viable can be caused to improve beyond the state to which they have degenerated and accordingly some improvement of function will be seen in patients.

In fed animals and in man the liver content, which is essentially that of blood, of acetoacetate is very low, eg. 0.09 mM and (R)-3- hydroxybutyrate is 0.123mM but rises after a 48 hour fast to eg. 0.65 mM acetoacetate and 1.8 mM (R)-3-hydroxybutyrate (84). The ketone bodies rise in starvation because the fall in insulin decreases the re-esterification of fatty acids to triglyceride in adipose tissue causing the release of free fatty acids into the blood stream. The released free fatty acids can then be taken up and used as a source of energy by muscle, heart, kidney and liver in the process of β-oxidation. Liver, however, has the capacity to convert the free fatty acids to a metabolic fuel, ketones, for use by extra-hepatic organs, including the brain, as an alternative to glucose during periods of fasting. The hepatic synthesis of ketone bodies occurs from mitochondrial acetyl CoA generated during the β-oxidation of fatty acids by liver.

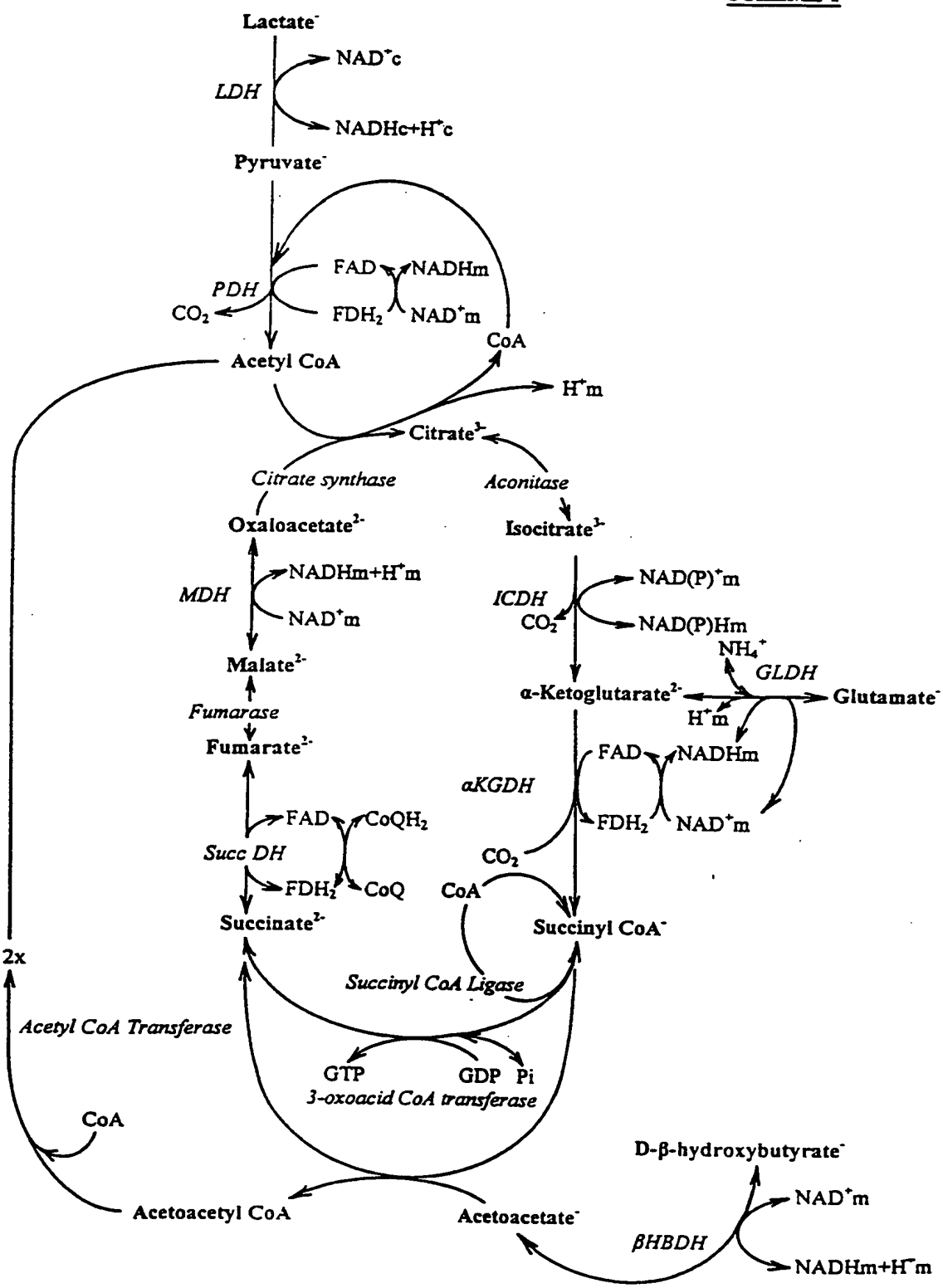
The ketone bodies enter extra-hepatic tissues on the same carrier, where other monocarboxylates can act as competitive inhibitors. Unphysiological isomers such as D-lactate or (S)-3-hydroxybutyrate can also act as competitive inhibitors to ketone body transport. Since ketone body transport across the blood brain barrier is a limiting factor to ketone body utilization in brain (76) every effort should be made to keep the blood concentration of these unphysiological enantiomers at low levels during ketogenic therapy. When blood ketone body concentrations are elevated to levels found in starvation, heart, muscle, kidney and brain utilize ketone bodies as the preferred energy substrate:

The present inventor has thus determined that the mitochondrial acetyl CoA derived from ketone bodies as produced using the cyclic oligomers taught by the present invention can thus replace the acetyl CoA deficiency which occurs during inhibition of PDH multienzyme complex in tissues dependent upon the metabolism of glucose for their supply of metabolic energy. The mitochondrial citrate supplied can also be transported to cytoplasm by the tri or dicarboxcylic acid transporter where it can be converted to cytoplasmic acetyl CoA required for the synthesis of acetyl choline. The reactions of the Krebs cycle are shown in Scheme 1 to help illustrate these concepts further.

Ketone bodies, in contrast to free fatty acids, cannot produce acetyl CoA in liver. Since acetyl CoA is the essential precursor of fatty acid, they cannot result in either increased fatty acid or cholesterol synthesis in liver, which usually accounts for over half of the body's synthesis of these two potentially pathogenic materials. Liver is sensitive to the ratio of acetoacetate /(R)-3-hydroxybutyrate presented to it and will alter its mitochondrial free $[NAD^+]/[NADH]$, because of the near equilibrium established by β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (31).

Inter alia, the aforementioned also indicates that one can provide a method of increasing the efficiency of mitochondrial energy production in a human or animal not suffering from a chronic or acute metabolic disease comprising administering to the human or animal an amount of a cyclic oligomer of formula (I) sufficient to raise blood levels of (R)-3-hydroxybutyrate to from 0.5 to 20mM.

SCHEME 1


Lactate⁻
NAD⁺c
LDH
NADHc+H⁺c
Pyruvate⁻
FAD
NADHm
PDH
CO₂
FDH₂
NAD⁺m
CoA
Acetyl CoA
H⁺m
Citrate³⁻
Citrate synthase
Aconitase
Oxaloacetate²⁻
Isocitrate³⁻
NADHm+H⁺m
MDH
NAD⁺m
NAD(P)⁺m
ICDH
CO₂
NAD(P)Hm
NH₄⁺
GLDH
Malate²⁻
α-Ketoglutarate²⁻
Glutamate⁻
H⁺m
Fumarase
Fumarate²⁻
FAD
NADHm
αKGDH
FAD
CoQH₂
FDH₂
NAD⁺m
Succ DH
CO₂
FDH₂
CoQ
CoA
Succinyl CoA⁻
Succinate²⁻
2x
Succinyl CoA Ligase
Acetyl CoA Transferase
GTP
GDP
Pi
3-oxoacid CoA transferase
CoA
D-β-hydroxybutyrate⁻
NAD⁺m
Acetoacetyl CoA
Acetoacetate⁻
βHBDH
NADHm+H⁺m

The easiest way to increase blood ketones is starvation. On prolonged fasting blood ketones reach levels of 7.5 mM (62, 63). However, this option is not available on a long term basis, since death routinely occurs after a 60 day fast.

The ketogenic diet, comprised mainly of lipid, has been used since 1921 for the treatment of epilepsy in children, particularly myoclonic and akinetic seizures (109) and has proven effective in cases refractory to usual pharmacological means (71). Either oral or parenteral administration of free fatty acids or triglycerides can increase blood ketones, provided carbohydrate and insulin are low to prevent re-esterification in adipose tissue. Rats fed diets comprised of 70% corn oil, 20% casein hydrolysate, 5% cellulose, 5%McCollum's salt mixture, develop blood ketones of about 2 mM. Substitution of lard for corn oil raises blood ketones to almost 5 mM (Veech, unpublished).

In general the levels of ketone bodies achieved on such diets are about 2 mM (R)-3-hydroxybutyrate and 1 mM acetoacetate while the levels of free fatty acids are about 1 mM. Other variations of composition have been tried including medium chain length triglycerides. In general, compliance with such restricted diets has been poor because of their unpalatability (56). High lipid, low carbohydrate diets also have been tried as therapeutic agents in cancer patients to reduce glucose availability to tumors (88), as weight reducing diets in patients with and without diabetes (74, 112) and to improve exercise tolerance (83).

The limitation of diets which rely upon lipid to raise blood ketones to neurologically effective levels are many. Firstly, levels of ketone bodies on lipid based diets tend to be below 3 mM, significantly lower than the level of 7.5 mM achieved in overweight humans during prolonged fasting. Secondly, unauthorized ingestion of carbohydrate increases insulin secretion and causes a rapid decrease in the hepatic conversion of free fatty acids to ketones with a consequent drop in blood ketones and the diversion of lipid to esterified to triglycerides by adipose tissue. Many anecdotal reports relate the resumption of seizures in children who "broke their diet with birthday cake". Thirdly their unpalatability and the necessity to avoid carbohydrate to sustain high ketone body levels makes such high lipid diets difficult to use in adults in an out patient setting, particularly in societies where traditionally high intake of refined sugars, bread, pasta, rice and potatoes occurs. In practice, the traditional high ketone diet

cannot be enforced in patients, other than children beyond the age where all food is prepared at home under strict supervision. Fourthly, ingestion of such large amounts of lipid in the adult population would lead to significant hypertriglyceridemia and hypercholesterolemia with pathological sequelae of increased vascular disease and sporadic hepatic and pancreatic disease, and therefore could not be prescribed on medical grounds. Ingestion of high lipid, low carbohydrate diets were popular in the 1970s for weight reduction in the face of high caloric intake, provided that carbohydrate intake was low. However, because of the increased awareness of the relationship of elevated blood lipids to atherosclerosis the popularity of this diet dropped abruptly.

Substituting glucose in a liquid diet, where glucose accounts for 47% of the caloric content, with racemic 1,3 butandiol caused the blood ketone concentration to rise about 10 fold to 0.98mM (R)-3-hydroxybutyrate and 0.33 mM acetoacetate (107). These values are slightly less than obtained normally in a 48 hour fast and far below the levels of 7.5 mM obtained in fasting man . Racemic 1,3 butandiol is converted by liver to acetoacetate and both the unnatural L-β and the natural D-β-hydroxybutyrate (respectively (S) 3-hydroxybutanoate and (R)-3-hydroxybutyrate). Although racemic 1,3 butandiol has been extensively studied as a cheap caloric source in animal food and has even been used experimentally in human diets (81, 101) the production of the unnatural L-isomer is likely in the long run to produce significant toxicity as has been shown for the human use of the unnatural D-lactate (64). One disadvantage of administering the unnatural L isomer is that it competes for transport with the natural (R)-3-hydroxybutyrate. Thus provision of the (R) 1,3 butandiol as a precursor of ketone bodies is one possibility that avoids unnecessary administration or production of the unnatural isomer.

The mono and di-aceotacetyl esters of racemic 1,3 butandiol have been suggested as a source of calories and tested in pigs (67). Oral administration of a bolus of a diet containing 30% of calories as the esters produced a brief peak of blood ketones to 5 mM. However, the use of racemic 1,3 butandiol with its production of the abnormal (S) 3-hydroxybutanoate is not to be recommended for the reasons stated above.

While use of racemic 1,3 butandiol in such formulations is not recommended, the esters of (R) 1,3 butandiol can be used, either alone or as the acetoacetate ester.

Studies in rats have shown that feeding racemic 1,3 butandiol caused liver cytosolic [NAD']/[NADH] to decrease from 1500 to about 1000 (87). By comparison, administration of ethanol reduces hepatic [NAD-]/[NADH] to around 200 (106).

Acetoacetate, when freshly prepared, can be used in infusion solutions where it can be given in physiologically normal ratios with (R)-3-hydroxybutyrate to optimum effect (95). Because of manufacturing requirements which currently require long shelf life and heat sterilized fluids, acetoacetate has frequently been given in the form of an ester. This has been done to increase its shelf life and increase its stability to heat during sterilization. In the blood stream, esterase activity has been estimated to be about 0.1 mmol/min/ml and in liver about 15 mmol/min/g (68). In addition to esters combining 1,3 butandiol and acetoacetate there has also been extensive study of glycerol esters of acetoacetate in parenteral (59) and enteral nutrition (82). Such preparations were reported to decrease gut atrophy, due to the high uptake of acetoacetate by gut cells and to be useful in treatment of burns (85).

For preferred embodiments of the present invention, under optimum conditions, a physiological ratio of ketones should be produced through administration of cyclic oligomers and acetoacetate. If it is not, in the whole animal, the liver will adjust the ratio of ketones in accordance with its own mitochondrial free [NAD^+]/[NADH]. If an abnormal ratio of ketones is given the liver will adjust the ratio, with coincident changes in liver [NAD^+]/[NADH]. In the working heart, perfusion with acetoacetate as sole substrate, rapidly induces heart failure (99) in contrast to rat hearts perfused with a mixture of glucose, acetoacetate and (R)-3-hydroxybutyrate, where cardiac efficiency was increased by a physiological ratio of ketone bodies (95).

The cyclic oligomers for use in the present invention are conveniently synthesized from the microorganism produced polyesters. Natural polyesters of (R)-3-hydroxybutyrate are sold as articles of commerce eg. as polymers of 530,000 MW from Alcaligenes eutrophus (Sigma Chemical Co. St. Louis) or as 250,000 MW polymers for sugar beets (Fluka, Switzerland). The bacteria produce the polymer as a source of stored nutrient. The fermentation of these polymers by bacteria was developed in the 1970s by ICI in the UK and Solvay et Cie in Belgium, as a potentially biodegradable plastic for tampon covers and other uses. The system responsible for the synthesis of the poly (R)-3-hydroxybutyrate has now been cloned and variations in the composition

of the polymer produced, based on the substrates given to the bacteria. The genes responsible for the synthesis of polyalkanoates have been cloned and expressed in a number of micro-organisms (93, 102, 113) allowing for production of this material in a variety of organisms under extremely variable conditions.

Preferred forms of cyclic oligomeric (R)-3-hydroxybutyrate are, at least in part, readily digestable and/or metabolised by humans or animals. These preferably are of 2 to 200 repeats, typically 2 to 20 and most conveniently from 3 to 10 repeats long, particularly of 3 repeats, ie. the triolide. It will be realised that mixtures of such oligomers may be employed with advantage that a range of uptake characteristics might be obtained. Similarly mixtures with the monomer or linear oligomers or polymers may be provided in order to modify the blood level profile produced.

Cyclic oligomers for use in the invention may be provided, inter alia, by methods described by Seebach et al. Helvetia Chimica Acta Vol 71 (1988) pages 155-167, and Seebach et al. Helvetia Chimica Acta, Vol 77 (1994) pages 2007 to 2033. For some circumstances such cyclic oligomers of 5 to 7 or more (R)-3-hydroxybutyrate units may be preferred as they may be more easily broken down in vivo. The methods of synthesis of the compounds described therein are incorporated herein by reference.

Once the monomer is in the blood stream, and since liver is incapable of metabolizing ketone bodies but can only alter the ratio of (R)-3-hydroxybutyrate/acetoacetate, the ketone bodies are transported to extrahepatic tissues where they can be utilized. The blood levels of ketones achieved are not subject to variation caused by noncompliant ingestion of carbohydrate, as is the case with the present ketogenic diet. Rather, they would simply be an additive to the normal diet, given in sufficient amounts to produce a sustained blood level, typically of between 0.3 to 20mM, more preferably 2 to 7.5 mM, over a 24 hour period, depending upon the condition being treated. In the case of resistant childhood epilepsy, blood levels of 2 mM are currently thought to be sufficient. In the case of Alzheimer's disease, attempts could even be made to keep levels at 7.5 mM or more, as achieved in the fasting man studies, in an effort to provide alternative energy and acetyl CoA supplies to brain tissue in Alzheimer's patients where PDH capacity is impaired because of excess amounts of $A\beta_{1-42}$ amyloid peptide (77, 78).

The determination by the inventor that (R)-3-hydroxybutyrate and its mixtures with acetoactetate act as a nerve stimulant, eg. nerve growth stimulant and/or stimulant of axon and dendritic growth, opens up the option of raising ketone body levels to lesser degrees than required nutritionally in order to treat neurodegeneration.

Compositions of the invention are preferably sterile and pyrogen free, particularly endotoxin free. Secondly, they are preferably formulated in such a way that they can be palatable when given as an additive to a normal diet to improve compliance of the patients in taking the supplements. The cyclic oligomers are generally smell free. Formulations of the cyclic oligomers of (R)-3-hydroxybutyrate and its mixtures with acetoacetate may be coated with masking agents or may be targeted at the intestine by enterically coating them or otherwise encapsulating them as is well understood in the pharmaceuticals or nutraceuticals art.

Since ketone bodies contain from about 4 to 6 calories/g, there is preferably a compensatory decrease in the amounts of the other nutrients taken to avoid obesity.

Particular advantages of using the cyclic oligomers taught in the present invention are:

1) they can be eaten with a normal dietary load of carbohydrate without decreasing blood ketone body levels which decrease would impair the effects of the treatment,

2) they will not raise blood VLDL and cholesterol, as with current cream and margarine containing diets, thus eliminating the risk of accelerated vascular disease, fatty liver and pancreatitis,

3) they will have a wider range of use in a greater variety of patients, including but not limited to: type II diabetes to prevent hypoglycemic seizures and coma, in Alzheimer's disease and other neurodegenerative states to prevent death of nerve cells eg. hippocampal cells, and in refractory epilepsy due to either decreases in cerebral glucose transporters, defects in glycolysis, or so called Leigh's syndromes with congenital defects in PDH.

The cyclic oligomers of the invention can be used in oral and parenteral use in emulsions, whereas acetoacetate, in the unesterified state, is less preferred as it is subject to spontaneous decarboxylation to acetone with a half time at room temperature

of about 30 days. Where the compositions of the invention do include acetoacetate this may be in the form of a precursor. Acetoacetate may conveniently be provided as (R)-3-hydroxybutyrate esters as provided by the copending 'therapeutic compositions' application.

Treatment may comprise provision of a significant portion of the caloric intake of patients with the cyclic (R)-3-hydroxybutyrate oligomer or oligomers formulated to give retarded release, so as to maintain blood ketones in the elevated range, eg. 0.5 to 20 mM, preferably 2-7.5 mM range, over a 24 hour period. Release of the ketone bodies into the blood may be restricted by application of a variety of techniques such as microencapsulation, adsorption and the like which is currently practised in the oral administration of a number of pharmaceutical agents. Enterically coated forms targeting delivery post stomach may be particularly used where the material does not require, or is not susceptible to, hydrolysis in acid environment. Where some such hydrolysis is desired uncoated forms may be used. Some forms may include enzymes capable of cleaving the esters to release the ketone bodies such as those referred to in Doi. Microbial Polyesters.

Preferred cyclic oligomers, eg. the triolide, may be merely added as such to foodstuffs and/or may be supplemented in a treatment regime by other ketone body generators of different release profile such as the monomeric (R)-3-hydroxybutyrate. The latter can be provided as an aqueous solution, eg. as a salt, eg. sodium, potassium, magnesium or calcium salt

For a 1500 calorie diet, the human adult patient could consume 198 g of cyclic esters of the present invention per day. For a 2000 calorie diet of the same proportions, one could consume 264 g of ketones per day. On the ketogenic lipid diet blood ketones are elevated to about 2 mM, which proves to be effective to some degree at least in over 60% of children treated. On the ketone diet, ketone levels should be higher because ketones have been substituted at the caloric equivalent of fat, that is 1.5 g of ketone/ g of fat. Accordingly, blood ketones should be approximately 3 mM, an effective level in children, but still below the level achieved in fasting man of 7.5mM.

The advantage of using compounds which directly raised ketone body levels, including the present cyclic oligomers which raise blood levels of ketone bodies themselves are several. Firstly, provision of ketone bodies themselves does not require

the limitation of carbohydrate, thus increasing the palatability of the dietary formulations, particularly in cultures where high carbohydrate diets are common. Secondly, ketone bodies can be metabolised by muscle, heart and brain tissue, but not liver. Hence the fatty liver, which may be an untoward side effect of the ketogenic diet, is avoided. Thirdly, the ability to include carbohydrate in the dietary formulations increases the chance of compliance and opens up practical therapeutic approaches to type II diabetics where insulin is high, making the known ketogenic diet unworkable.

The present inventor has determined that, while any elevation of ketone bodies may be desirable, a preferred amount of cyclic ester to be administered will be sufficient, with any acetoacetyl component, to elevate blood ketone body levels to the 0.5 to 20mM level, preferably to the 2mM to 7.5mM level and above, particularly when attempting to arrest the death of brain cells in diseases such as Alzheimer's and Parkinsonism. While dead cells cannot be restored, arrest of further deterioration and at least some restoration of function is to be anticipated.

The total amount of ketone bodies used in treatment of neurodegeneration such as Alzheimer's and Parkinsonism will preferably elevate blood levels of ketone bodies by from 0.5mM to 20mM. The present inventor estimates that 200 to 300g (0.5 pounds) of ketone bodies equivalent per patient per day might be required to achieve this. Where the treatment is through maintenance of cells against the effects of neurotoxin this may be at a level sufficient to act as a significant caloric source, eg. 2 to 7.5mM in blood. Where it relies on the nerve stimulatory factor effect of the (R)-3-hydroxybutyrate so produced, the amount administered may be lower, eg. to provide 0.2 to 4 mM increase, but can of course be more for this or other disease.

It will be realised that treatment for neurodegenerative diseases such as Alzheimer's or Parkinsonism will most effectively be given soon after identifying patient's with a predisposition to develop the disease. Thus treatment for Alzheimers' most effectively follows a positive test result for one or more conditions selected from the group (i) mutations in the amyloid precursor protein gene on chromosome 21, (ii) mutations in the presenilin gene on chromosome 14, (iii) presence of isoforms of apolipoprotein E. Other tests shown to be indicative of Alzheimer's will of course be applicable.

Following such a positive test result it will be appropriate to prevent the development of memory loss and/or other neurological dysfunction by elevation of the total sum of the concentrations of the ketone bodies (R)-3-hydroxybutyrate and/or acetoacetate in the patient's blood or plasma to say between 1.5 and 10 mM, more preferably 2 to 8mM, by one of several means. Preferably the patient is fed a diet of sufficient quantities of compound of formula (I), optionally parenterally but preferably and advantageously enterally.

It will be realised that hypoglycemic brain dysfunction will also be treatable using the treatments and compositions and compounds of the present invention. A further property associated with the present treatment will be general improvement in muscle performance.

The provision of cyclic oligomer based foodstuffs and medicaments of the invention is faciliated by the ready availability of a number of relatively cheap, or potentially cheap, starting materials from which cyclic (R)-3-hydroxybutyric acid may be derived (see Microbial Polyesters Yoshiharu Doi. ISBN 0-89573-746-9 Chapters 1.1, 3.2 and 8). The availability of genes capable of insertion into foodstuff generating organisms provides a means for creating products such as yoghurts and cheese that are enriched in the cyclic oligomer-(R)-3-hydroxybutyric acid or, after breakdown with enzymes capable of cleaving such polymers, with the monomeric substance itself (see Doi. Chapter 8).

Methods of preparing poly (R)-3-hydroxybutyrate are not specifically claimed as these are known in the art. For example Shang et al, (1994) Appli. Environ. Microbiol. 60: 1198-1205. This polymer is available commercially from Fluka Chemical Co. P1082, cat#81329, 1993-94, 980. Second St. Ronkonkoma NY 11779-7238, 800 358 5287.

The present invention will now be described further by way of illustration only by reference to the following Figures and experimental examples. Further embodiments falling within the scope of the invention will occur to those skilled in the art in the light of these.

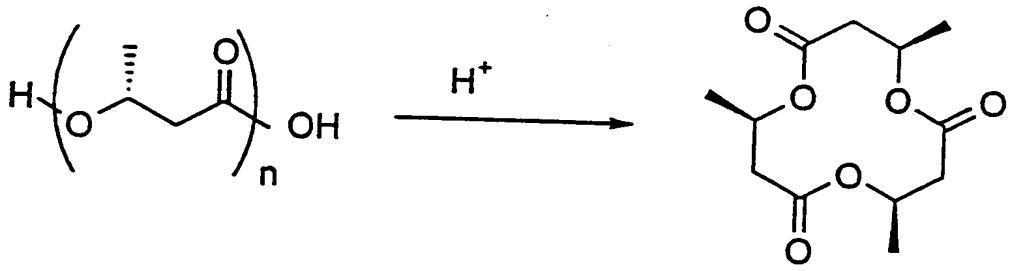
FIGURE

Figure 1 is a graph showing blood (R)-3-hydroxybutyrate level produced after time after feeding rats with the triolide of (R)-3-hydroxybutyrate, a cyclic oligomer produced in Example 1 in yoghurt and controls fed yoghurt alone.

EXAMPLES

Example 1.

Preparation of (R,R,R)-4, 8, 12-trimethyl-1, 5, 9-trioxadodeca-2, 6, 10-trione: triolide of (R)-3-hydroxybutyric acid.



Synthesis was as described in Angew. Chem. Int. Ed. Engl. (1992), 31, 434. A mixture of poly[(R)-3-hydroxybutyric acid] (50g) and toluene-4-sulphonic acid monohydrate (21.5g, 0.113 mole) in toluene (840ml) and 1,2-dichloroethane (210ml) was stirred and heated to reflux for 20 hours. The water was removed by Dean-stark trap for 15 hours whereafter the brown solution was cooled to room temperature and washed first with a half saturated solution of sodium carbonate then with saturated sodium chloride, dried over magnesium sulphate and evacuated in vacuo. The brown semi-solid residue was distilled using a Kugelrohr apparatus to yield a white solid (18.1g) at 120-130°C/0.15mmHg. Above 130°C a waxy solid began to distill – distillation being stopped at this point. The distilled material had mp 100-102°C (literature mp 110-110.5°C). Recrystallisation from hexane gave colourless crystals in yield 15.3g. Mp=107-108°C; $[\alpha]_D$-35.1 (c=1.005, $CHCl_3$), (lit. = -33.9). ^1H NMR (300 MHz, $CDCl_3$): δ=1.30 (d, 9H, CH_3); 2.4-2.6 (m,6H; CH_2); 5.31-5.39 (M, 3H; HC-O). ^{13}C NMR ($CDCl_3$) δ=20.86 (CH_3), 42.21 (CH_2), 68.92 (CH), 170.12 (CO).

Elemental analysis: calculated for $C_{12}H_{18}O_6$: C, 55.81; H 7.02; Found: C, 55.67; H, 7.15.

Comparative Example 1: Preparation of oligomers of (R)-3-hydroxybutyric acid (R)-3-hydroxybutyrate).

(R)-3-hydroxybutyric acid (Fluka-5.0g: 0.048mole), p-toluene sulphonic acid (0.025g) and benzene (100ml) were stirred under reflux withn a Dean-Stark trap arrangement for 24 hours. The reaction mixture was cooled and the benzene evaporated in vacuo (0.5mm Hg). 4.4g of colourless oil was obtained of which a 20mg sample was converted to the methyl ester for analysis of number of monomer repeats using NMR. These studies show that the product is a mixture of oligomers of D-β-hydroxybutyrate of average number of repeats 3.75, being mainly a mixture of trimers, tetramers and pentamers with the single most abundant material being the tetramer. The product mixture was soluble in 1 equivalent of sodium hydroxide.

Comparative Example 2: Preparation of acetoacetyl ester of oligomeric (R)-3-hydroxybutyric acid.

A further batch of the colourless oil product from Example 1 (4.5g) was heated for 1 hour at 60°C with diketene (3.8g) and sodium actetate (0.045g) under nitrogen. Further diketene (3.8g) was added and the reaction heated for a further hour, cooled and diluted with ether, washed with water and then extracted with saturated sodium bicarbonate (5 x 100ml). Combined extract was washed with ether then acidified with concentrated HCl (added dropwise). Ethyl acetate extraction (3 x 50ml) was followed by drying over magnesium sulphate and evaporation *in vacuo*. A yellow solid/oil mixture was obtained (7.6g) which was chromatographed on a silica column using dichloromethane/methanol (98:2) to give a light amber oil product. Faster moving impurities were isolated (1.6g) and after recolumning carbontetrachloride/methanol (99:1) 0.8g of oil was recovered which was shown by NMR and Mass spectrometry to be the desired mixture of acetoacetylated oligomers of (R)-3-hydroxybutyrate. The product mixture had an Rf of 0.44 in dichloromethane/methanol (90:1) and was soluble in 1 equivalent of sodium hydroxide.

Both products of Comparative Examples 1 and 2 are amenable to separation of individual components by preparative HPLC.

Example 2.

Oral administration of triolide of (R)-3-hydroxybutyrate of Example 1 to rats.

The ability of orally administered triolide to raise blood ketone levels was investigated as follows. The day before the experiment commenced, 12 Wistar rats weighing 316±10g were placed in separate cages. They had no access to food for 15 hours prior to presentation with triolide containing compositions, but water was provided *ad libitum*.

On the morning of the experiment 0.64g of triolide was mixed with 5g Co-op brand Black Cherry yoghurt in separate feeding bowls for 9 of the rats. The remaining 3 rats were given 5g of the yoghurt without the triolide as controls. The yoghurt containing bowls were placed in the cages and the rats timed while they ate. Two of the three control rats ate all the yoghurt and four of the six triolide yoghurt rats ate approximately half the provided amount. The remaining six rats slept.

Control rats (n=2) were killed at 60 and 180 minutes after ingestion of yoghurt while triolide fed rats were killed at 80, 140, 150 and 155 minutes. Blood samples were taken for assay of (R)-3-hydroxybutyrate. Brains were funnel frozen and later extracted in perchloric acid and extracts neutralised and assayed. Blood levels of (R)-3-hydroxybutyrate were measured using a NAD^+/EDTA assay of Anal. Biochem (1983) 131, p478-482. 1.0ml of a solution made up from 2-amino-2-methyl-1-propanol (100mM pH 9.9, 0.094g/10ml), NAD^+ (30mM, 0.199g/10ml) and EDTA (4mM, 0.015g/10ml) was added to each of a number of cuvettes and 4µl sample or (R)-3-hydroxybutyrate control.

The two control rats ate 5.2±0.1g yoghurt and their plasma (R)-3-hydroxybutyrate concentrations were about 0.45mM at 60 minutes and 180 minutes. The four triolide fed rats ate 0.39±0.03g of the triolide and 2.6±0.2g of yoghurt. Their plasma (R)-3-hydroxybutyrate concentrations were 0.8mM after 80 minutes and 1.1mM for the group sacrificed at about 150 minutes. All rats displayed no ill effects from ingestion of triolide. Thus serum (R)-3-hydroxybutyrate was found to be elevated by 0.65mM by feeding of only 0.4g triolide. Note, as the rats had been fasted, the initial levels of (R)-3-hydroxybutyrate were elevated from the 0.1mM fed

state to about 0.45mM.

The test rats thus showed increase in plasma (R)-3-hydroxybutyrate over at least 3 hours with no ill effects. It should be noted that two other rats fed approximately 1.5g triolide each in 'Hob-Nob' biscuit showed no ill effects after two weeks.

It should be noted that the increased levels of (R)-3-hydroxybutyrate will also be mirrored in acetoacetate levels, not measured here, as there is a rapid establishment of equilibrium between the two in vivo such that acetoacetate levels will be between 40 and 100% of the (R)-3-hydroxybutyrate levels.

Comparative Example 3: Oral administration of (R)-3-hydroxybutyrate, oligomers and acetoacetyl (R)-3-hydroxybutyrate oligomers to rats.

The ability of orally administered (R)-3-hydroxybutyrate and the linear oligomers of Comparative examples 1 and 2 to raise blood ketone body levels was investigated as follows. Rats were fasted overnight and then gavaged with 100 µl/100g bodyweight of 4M (R)-3-hydroxybutyrate brought to pH 7.4 using methyl glucamine. Plasma levels of (R)-3-hydroxybutyrate were measured at 0.62mM after 30 minutes as compared to 3mM when 9M (R)-3-hydroxybutyrate is used.

This procedure was repeated with 2M solutions of the mixtures (R)-3-hydroxybutyrate oligomers and their acetoacetyl esters described in Comparative Examples 1 and 2. The (R)-3-hydroxybutyrate oligomer (19/1) and the acetoacetyl ester (20/4) were both brought to pH 7.6 with methyl glucamine and the blood (R)-3-hydroxybutyrate level monitored using the aforesaid assay procedure. Increases in serum (R)-3-hydroxybutyrate were shown to be of 0.2mM to 0.5mM at 60 and 120 minutes after gavaging.

Example 5.

Table 2. Sample 1500 calorie ketogenic diet using cyclic oligomer (I) of invention. The cyclic oligomer is assumed to contain 6 kcal/g fats, 9 kcal/g carbohydrate and 4 kcal/g protein. Oligomers have been substituted to give equivalent calories.

	Amount (g)	Fat (g)	Protein (g)	CHO (g)	Cyclic (I) (g)
Breakfast					
Egg	32	4	4		
apple juice	70			7	
ketones	66				66
skim milk	92	0	2	3	
Total Breakfast		4	6	10	66
Lunch					
lean beef	12	1.75	3.5		
cooked carrots	45		0.6	3	
canned pears	40			4	
ketones	69.75				69.75
skim milk	92		2	3	
Total Lunch		1.75	6.1	10	69.75
Supper					
Frankfurter	22.5	6	3		
cooked broccoli	50		1	2	
watermelon	75			5	
ketones	62.25				62.25
skim milk	92		2	3	
Total Supper		6	6	10	62.25
Daily Total		**11.75**	**18.1**	**30**	**198**

Example 6: Effect of (R)-3-hydroxybutyrate on hippocampal cells.

Methods

Culture Medium and Chemicals

The serum free medium used from 0 to day 4 contained Neurobasal medium with B27 supplement diluted 50 fold (Life Technology, Gaithersburg, MD) to which was added: 0.5 mM L-glutamine, 25 μM Na L-glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. After day 4, DMEM/F12 medium containing 5 μM insulin, 30 nM l-thyroxine, 20 nM progesterone, 30nM Na selenite 100 U/ml penicillin and 100 μg/ml streptomycin were used.

Hippocampal Microisland Cultures

The primary hippocampal cultures were removed from Wistar embryos on day 18 and dispersed by gentle agitation in a pipette. The suspension was centrifuged at 1,500 x g for 10 min and the supernatant discarded. The pellet was resuspended in new media to a final cell count of 0.4-0.5 x 10^6 cells/ml. Ten μl of this suspension was pipetted into the center of poly D-lysine coated culture wells and the plates incubated at 38°C for 4 hrs and then 400 μl of fresh Neurobasal media was added. After 2 days of incubation, half of the media was exchanged for fresh media and the incubation continued for 2 more days. After day 4, the medium was changed with DMEM/F12 medium containing 5μM insulin, 30 nM l-thyroxine, 20 nM progesterone, 30 nM Na selenite 100 U/ml penicillin and 100 μg/ml streptomycin. The wells were divided into 4 groups: half the wells received (R)-3-hydroxybutyrate to a final concentration of 8 mM while and half of the wells received 5 nM amyloid β_{1-42} (Sigma). These media were exchanged 2 days later (day 8) and the cells were fixed on day 10 and stained with anti MAP2 (Boehringer Manheim, Indianapolis IN) to visualize neurons and vimentin and GFAP (Boehringer) to visualize glial cells.

Results

Cell Counts

Addition of (R)-3-hydroxybutyrate to the incubation resulted in an increase in the neuronal cell number per microisland from a mean of 30 to a mean of 70 cells per microisland. Addition of 5 nM amyloid β_{1-42} to the cultures reduced the cell numbers from 70 to 30 cells per microisland, confirming the previous observations of Hoshi *et al*, that amyloid β_{1-42} is toxic to hippocampal neurons. Addition of (R)-3-

hydroxybutyrate to cultures containing amyloid β_{1-42} increased the cell number from a mean of 30 to 70 cells per microisland. From these data we conclude that addition of substrate level quantities of (R)-3-hydroxybutyrate, to media whose major nutrients are glucose, pyruvate and L-glutamine, slows the rate of cell death in culture. It is further concluded that (R)-3-hydroxybutyrate can decrease the increased rate of hippocampal cell death caused by the addition of amyloid β_{1-42} in culture.

The number of dendritic outgrowths and the length of axons were both observed to have increased with presence of(R)-3-hydroxybutyrate, whether β_{1-42} was present or not. This is indicative of nerve growth factor like behaviour.

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